Protein import into peroxisomes of Hansenula Polymorpha.

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Summary

PROTEIN IMPORT INTO PEROXISOMES OF *HANSENULA POLYMORPHA*

Eukaryotic cells are characterized by the presence of distinct subcellular compartments which are involved in specific metabolic processes. These compartments are collectively called organelles and include the nucleus, mitochondria, vacuoles and microbodies. Microbodies form a class of relatively recently discovered organelles (1950's), which can be subdivided in peroxisomes, glyoxysomes and glycosomes depending on their metabolic function. Microbodies consist of a protein-rich matrix surrounded by a single membrane. They lack DNA. The size and the number of microbodies per cell is influenced by the environmental conditions. This implies that microbodies are inducible in nature. The essential contribution of microbodies to cell viability is most dramatically demonstrated by an inheritable disease in human called Zellweger Syndrome, which is caused by peroxisomal disfunctioning. Patients suffering from this disease often die early after birth.

In yeasts, clear development of peroxisomes is observed when they are grown on certain specific substrates. Glucose-grown cells contain only 1 or 2 very small peroxisomes with a still unknown function. Oleic acid and methanol induce strong peroxisome proliferation in yeasts capable of growth on these carbon sources.

The organism studied in this thesis is the methylotropic yeast *Hansenula polymorpha*. During growth of this organism on methanol many peroxisomes are present, which contain the metabolic enzymes, alcohol oxidase (AO), dihydroxyacetone synthase (DHAS) and catalase (CAT). In addition, peroxisome proliferation is observed when the yeast is grown on specific N-sources, such as alkylated amines. Over the past 20 years, studies on peroxisome biogenesis in *H. polymorpha* have contributed much to our current knowledge about this class of organelles. Industrial interest in *H. polymorpha* arose when it became clear that methanol-grown cells synthesize the proteins involved in methanol metabolism, such as AO and DHAS, to very high levels. The intrinsically strong, methanol-inducible, transcription and translation machinery is of considerable potential for the production of heterologous proteins.

In this thesis two main subjects were studied: (1) import of proteins into the peroxisomes of *H. polymorpha* and (2) synthesis and accumulation in peroxisomes of heterologous small functional peptides (SFP's). 'Protein packaging' of these SFP's into microbodies was thought to prevent their rapid, intracellular, proteolytic degradation and/or protect the host cell from toxic effects caused by the SFP's.

Before *H. polymorpha* could be used for studying peroxisome biogenesis at the molecular level and for expression of foreign genes, basic tools for the genetic manipulation of this yeast had to be developed. Chapters 2 and 3 describe methods for the introduction and maintenance of foreign DNA in *H. polymorpha*. Electroporation appeared to be a most efficient procedure for transforming this yeast, yielding over 10^6 transformants per μg plasmid DNA. An increasing number of transformants was obtained when (1) the amount of DNA (up to 10 μg) and/or (2) the cell density of the electroporation mixture was increased,

making this procedure pre-eminently suitable when high numbers of transformants are required. Furthermore, transformation frequencies were improved by linearizing the plasmid DNA prior to transformation. Analysis of the transformants obtained revealed that the plasmids were present as circular molecules indicating that *H. polymorpha* possesses an efficient double-strand break repair system. These studies also showed that the selectable gene used in these plasmids, the *Saccharomyces cerevisiae LEU2*, contains sequences which promote autonomous replication of the plasmids in *H. polymorpha*. The same plasmids were also used for site-specific integration in the *H. polymorpha* genome. This was accomplished by linearizing the plasmids prior to transformation, which yields recombinogenic ends that share homology to genomic sequences.

Amine oxidase (AMO) is a peroxisomal protein which plays an crucial role in the metabolism of *H. polymorpha* when the yeast is grown in media containing alkylated amines as the sole nitrogen source. We have located the amino acid sequences in AMO which are essential for peroxisomal targeting. As a first possible candidate we have examined an SRL motif at nine amino acids from the C-terminus, which fits in the PTS1 consensus sequence S/A/C-K/R/H-L. Variants of this sequence are present at the extreme C-termini of many peroxisomal proteins and are shown to be essential for peroxisomal targeting. However, when a mutated AMO protein, in which the C-terminal sequence was changed from -SRLAFEGSCCGK to -SIETCL was synthesized in *H. polymorpha*, we observed normal targeting of the mutant AMO protein. This indicates that the SRL-containing C-terminal sequence is not essential for targeting (Chapter 4).

Subsequently, we focussed our attention on the N-terminal part of AMO. The AMO N-terminal sequence shows significant similarity to the targeting sequences found in peroxisomal thiolases (PTS2). Deleting the N-terminal 16 amino acids from AMO indeed abolished peroxisomal import. In addition, fusing the 16 N-terminal amino acids to a reporter protein led to the (partial) peroxisomal accumulation of the hybrid protein, indicating that this sequence indeed contains essential targeting information. Therefore, AMO is considered to be a PTS2 protein (Chaper 5).

Comparable studies were performed with a heterologous protein, glyoxysomal malate dehydrogenase (gMDH) from watermelon. Like the peroxisomal thiolases from rat, this protein contains an N-terminal presequence which is proteolytically processed upon import into glyoxysomes. We synthesized this protein in *H. polymorpha* and studied its subcellular destination. During methylotrophic growth of transformed cells, pre-gMDH was imported into peroxisomes. The apparent molecular mass of the protein was equal to that of pre-gMDH, implying that N-terminal processing of the transit peptide had not occured in *H. polymorpha* (Chapter 6). Subsequently, it was shown that the 37-amino acid N-terminal transit peptide is capable to target a reporter protein, consisting of the mature part of watermelon mitochondrial malate dehydrogenase, to peroxisomes. This peptide contains the motif, RI-X_5-HL, which resembles the PTS2 consensus sequence RL-X_5-H/QL. Mutational analysis revealed that these amino acids are indeed essential for proper targeting whereas

amino acid substitutions outside this sequence did not interfere with subcellular sorting (Chapter 7).

As suggested by the occurence of different targeting signals, distinct import pathways for peroxisomal proteins exist. Moreover, the import pathway for PTS2 proteins in *H. polymorpha* is inducible by specific growth conditions. This is concluded from results presented in Chapter 8. High-level synthesis of PTS2 proteins (AMO, watermelon gMDH or *S. cerevisiae* thiolase) in methanol-grown *H. polymorpha* cells, was obtained by placing their cognate genes under the control of the alcohol oxidase promoter. Efficient import of these proteins into peroxisomes was only observed if the transformed cells, were grown in the presence of specific N-sources, which in the wild-type host induce the synthesis of AMO.

Finally, as described in Chapter 9, *H. polymorpha* was used for the synthesis of heterologous small functional peptides (SFP's). Human Insulin-like Growth Factor-II (67 aa) and *Xenopus laevis* magainin II (23 aa) were synthesized as larger hybrid proteins, fused to the C-terminus of the peroxisomal carrier protein AMO. Especially in the case of the antimicrobial peptide magainin-II, this stratagy was chosen while production in its mature form is thought to be impossible. High-level synthesis of the hybrid proteins, exceeding 20% of total cellular protein, was obtained when the transformed strains were grown in methanol-limited chemostat.cultures. After partial purification, the hybrid proteins could be cleaved by proteolytic treatment with factor Xa, yielding the AMO-carrier and the SFP. Unexpectedly, peroxisomal accumulation of the fusion protein did not occur, indicating that import of these proteins via the PTS2 import pathway is impeded.